ANTIGENIC PROPERTIES OF EUGLENA GRACILIS

and certain other

Unicellular Chlorophyll-Bearing Organisms

by

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A. B. , William Jewell College, 1925

Submitted to the Department of
Bacteriology and the Faculty
of the Graduate School of the
University of Kansas in partial
fulfillment of the requirements
for the degree of Master of Arts

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August 20 - 1927

Acknowledgement

I gratefully acknowledge my indebtedness to Dr. Noble P. Sherwood, who has encouraged and directed throughout my interest in the antigenic properties of these organisms.

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INTRODUCTION

The problem of the antigenic properties of unicellular chlorophyll-bearing organisms was suggested by the work of Sherwood [25] in which he attempted to immunize rabbits to the chloroplasts of several higher plants. The serum of the treated animals did not bring about release of the chlorophyll from the chloroplasts in the presence of fresh serum or produce any changes detectable microscopically, and agglutinin production was inconstant. It was thought that a thorough immunological study of some simple chlorophyll-bearing organism might further our knowledge of the antigenic properties of chlorophyll in the chloroplast and, in addition, give valuable information about a group which has been insufficiently studied from this standpoint.

REVIEW OF LITERATURE

Although immunological work with plant material (other than bacteria) was undertaken at least as early as 1901 (Kowarski [11]), the first published account of use of algae seems to be that of Rosenblat-Lichtenstein [20] in 1912. She used pure cultures of

six species of Chlorophyceae: Chlorella protothecoides Krüger, Stichococcus sp. , and four others of uncertain identification. Rabbits were given many intravenous injections at intervals of five days or longer of several loopfuls of a culture grown upon glucose-peptone agar. The antisera produced were specific in proper dilution and had agglutinin titers of 1:3000 (Stichococcus), 1:3500 (Chlorella protothecoides), 1:2500 (Chlorella ?), and 1:10,000 (an organism of uncertain identification, probably a Chlorella) .

In a later paper [21] the same author compared the immune serum produced upon injection of the ordinary green Chlorella protothecoides with that produced upon injection of a colorless form of the same organism obtained by increasing the glucose content of the medium. A serum which agglutinated the homologous antigen in a dilution of 1:600 (colorless) and 1:400 (green) did not act upon the other form in a dilution of 1:100. A colorless culture which later became green was not affected by the colorless serum. Adsorption experiments showed that the agglutinins were distinct.

Lieske [13] found it necessary to use larger immunizing doses than those used by Rosenblat-Lichtenstein. He injected rabbits intraperitoneally and intravenously

every five to eight days for several weeks. The highest titered serum ever obtained agglutinated the homologous antigen in a dilution of 1:4000; most of the immune sera were much less active. Pure cultures grown upon an inorganic agar medium in the light or upon a malt-extract agar medium in the dark of fifteen species in twenty-three races were used. These included species of Chlorella, Stichococcus, Scenedesmus, Ancestrodesmus, Protococcus, Chlorococcum, Chlamydomonas, and Tetraspora. The author considered complement fixation more applicable to algal differentiation than agglutination, precipitation, or conglutination. He found a close antigenic relationship between Stichococcus bacillaris and Chlorella vulgaris. Pfeiffer's phenomenon could not be observed. Growth upon different media altered considerably the action of a species to an agglutinating serum. Because no serological difference could be detected between a green culture grown in the dark (organic medium) and a colorless form of the same organism, while differences could be detected between these and green cultures grown in the light (either organic or inorganic medium), Lieske concluded that it was not the presence or absence of chlorophyll which was responsible for the serological difference

between green and colorless cultures of the seme species, but the autotrophic or heterotrophic mode of nutrition conditioned thereby.

Mez and his associates at the Königsberg Botanical Institute have been carrying out extensive serological investigations throughout the entire plant kingdom since 1911, from which they have drawn wide conclusions as to the evolutionary development and systematic position of the forms studied. Work on the algae, published by Steinecke [26] in 1925, is of interest in this connection. It is, as far as I know, the only immunological work ever reported in which euglenas were used, even under the handicap of impure material. Precipitation and conglutination were studied with immune rabbit serum, using as antigen protein extract of many forms (among them Euglena viridis) prepared from dried material collected from ponds. The following conclusions were drawn:

Autotrophic bacteria were the first living things. The bacteria are related to the blue-green algae and have nothing to do with the fungi. The lower green algae developed from the blue-green algae. Two great groups of green flagellates must be considered. One (Volvocales) has developed directly from the green algae. The other represents reproductive cells of the higher

green algae which have developed into independent organisms. This latter group of flagellates forms the basis for the animal kingdom. The animal kingdom, then, does not go back to the amebas, but to the higher algae. The euglenas, belonging to this flagellate group, represent intermediate forms between plants and animals.

Because the type of immunity produced to euglenas seems in some respects analogous to that produced to undoubted protozoa, mention should be made here of a few papers dealing with these latter forms. There is sufficient evidence that pathogenic protozoa are antigenic. The free-living ones have been less studied, and, as Calkins [4] states concerning the work of Rössle, the results obtained are "not convincing because of the impossibility of getting these organisms free from bacteria".

Rössle [19] in 1905 reported upon the immunity produced by injection of Paramecium and Glaucoma into rabbits. He described a specific toxic action, which consisted in a paralysis involving chiefly the cilia of the upper surface, preceded by a stage of lively motion with rapid rotation. If it extended no further than this recovery took place in from three to five days. The recovered organisms were not again affected

by the same serum dilution. True cytolysis probably did not occur, and inactivation of the serum did not alter its paralyzing properties. The ciliates sank to the bottom upon paralysis of movement. Another phenomenon also occurred, which Rössle interpreted as analogous to agglutination: adherence of the organisms to the walls of the test tube, or other solid bodies in their vicinity.

Coca [5] found complement fixation and agglutination satisfactory with amebas, but could not observe any specific cytotoxic influence on the part of the immune serum. Sellards [24], working with amebas, had demonstrated specific cytolytic serum active in low dilution. Heathman [10], working in this laboratory, carried out various serological reactions with amebas, and found these organisms highly antigenic.

In the identification of herpetomonads and leishmanias, where morphological differentiation is subject to error, Noguchi [16] found that agglutination and complement fixation confirm the results of fermentation tests.

Taliaferro [27] has recently presented a comprehensive review of the immunity produced in trypanosome infections, including his own work. The resistance, as studied in rats infected with Trypanosoma lewisi, is

manifested in three distinct ways. (1) In the first stage the chief property of the immune serum seems to be the presence of a "reaction product" which has no effect on the vitality of the trypanosomes but by the tenth day of infection totally inhibits their reproduction. It is this reproduction-inhibiting property of the serum rather than the lysin described below which is the basis of the permanent immunity. Taliaferro has attempted to compare this serum property with other known immune bodies. It can be passively transferred. It is non-ether soluble and is precipitated with the globulin fraction of the immune serum, coming down equally with the eu- and pseudoglobulin. "It differs from others, however, in its lack of in vitro affinity for its supposed antigen. Thus, when serum containing it is left in contact for twelve hours with large numbers of dividing Trypanosoma lewisi and the parasites subsequently removed, the serum does not lose any of its titer." (2) From the eighth to the twelfth days a sudden destruction of the majority of the parasites takes place. No explanation is given for this first crisis. (3) There occurs an eventual total destruction of the parasites which terminates the infection, and may take place from a few days to several months after the first crisis. Concerning this destruction,

Taliaferro says: "The available evidence indicates that at the crisis there is a sudden acquisition of a trypanolytic property by the blood serum and that the organisms accumulate in the blood during the relapse not because the lytic antibody disappears, but because the trypanosomes are so changed biologically as to be no longer susceptible to the action of the antibody." This lytic antibody is specific in proper dilution, and complement is necessary for its action.

The acquired resistance of the trypanosomes to the lytic antibody is such that a relapse strain is unaffected by the serum active against the passage strain, and the original passage strain is made resistant to the lytic antibody by a few minutes contact with the immune serum in vitro. Acquired resistance to injurious substances is a wide-spread phenomenon among the protozoa, and in all cases in which it has been studied it has been shown that it occurs within a single-cell strain, that it is inherited for many generations, though lost in time, and that it is usually associated with the destruction of a large part of the population. No convincing theory of the formation of antibody-resistant races has been presented. Ehrlich [7] assumed that the "nutriceptors" of the original strain combined with the antibody and atrophied, whereupon new receptors

developed which would not combine. Levaditi and McIntosh [12] believed that it was a case of natural selection with survival of the more resistant. Rosenthal [22] found that the trypanocidal property of immune serum does not vary proportionately with its power to produce relapse strains; and that the property of producing relapse strains is thermolabile and is recovered with the albumin fraction of the serum, whereas the trypanocidal property is not altered by heat and is recovered with both the albumin and globulin fractions. This work has not been verified.

EXPERIMENTAL WORK

Introduction

The organism selected for most extensive investigation was Euglena gracilis Klebs. To my knowledge no immunological study of any species of the order Euglenida (Stein) has heretofore been made, with the exception of the recent work of Steinecke, referred to above. This is surprising in view of their wide distribution and abundance in nature, their disputed systematic position, and their general use as type forms to illustrate organisms intermediate between the

plant and animal kingdoms. It is of particular interest to inquire into the antigenic constitution of such a group.

The analysis of the antigenic capacity of the forms used resolved itself into a study of the following:

1. The occurrence and nature of chlorolysins in normal and immune sera.

2. The presence of agglutinins in normal and immune sera.

3. The occurrence and nature of cytotoxins in normal and immune sera.

4. The presence of complement fixing substances in the immune sera.

5. The production of precipitins.

6. The demonstration of clinical anaphylaxis, of the smooth muscle reaction of Dale, and of the phenomenon of Arthus.

Materials and Methods

Pure cultures of two races of Euglena gracilis of widely different origin were obtained free from bacteria by single cell isolation with the Barber [1] technique. One of these, isolated on October 5,1926, and designated as the Noland strain, was from a laboratory culture obtained at the University of

Wisconsin; the other, isolated in March 1927, and designated as the Turtox strain, was from a culture purchased from the Chicago Biological Supply House.

The following medium, which is a modification of those recommended by Zumstein [31] and Pringsheim [18] for this species, was found the most satisfactory of those tried:

Peptone (Merck)	0.25 %
$MgSO_4$	0.05 %
KH_2PO_4	0.05 %
$(NH_4)_2SO_4$	0.10 %
$CaCO_3$ and $FeCl_3$ (traces)	0.0005 % each
Distilled water	

The reaction was not adjusted; the medium was filtered through absorbent cotton and sterilized in the autoclave. Stock cultures were kept in test tubes, and 250 cc. and 2 l. Erlenmeyer flasks were inoculated from these.

Growth in this medium, in the light of a north window at room temperature, was such that a month old culture yielded approximately one cubic centimeter of packed cells per liter of medium. The euglenas did not produce their green chlorophyll during the first few days of growth in this medium, and upon continued cultivation in it some reduction in the amount of chlorophyll produced took place.

The method used for concentration of a culture was suggested by an article by Bills [3]. The culture

flask was packed in ice over night. The euglenas settled to the bottom, making it possible to decant most of the medium.

The highly saprophytic tendency of Euglena gracilis has been emphasized by others [31, 28, 18] who have studied it in pure culture. Although it thrives best in the light with mixotrophic nutrition, it can grow nearly as well in the dark in a suitable organic medium, under which condition it is perfectly colorless. Accordingly, cultures of the Noland strain were carried through several transfers in the dark, and this colorless (packed cells yellowish) form compared with the green form in the studies to follow.

In addition to this one species of Euglena, the following unicellular algae were employed also, because of their ready growth in pure culture, and for purposes of comparison. A species of Chlorella (a) and one of Oocystis (b) were obtained in pure culture by plating in agar; the others* available were Stichococcus sp. (W2), Protosiphon botryoides (W3), two species of Chlorella (W5, W11), one species of Oocystis ? (W9), and Chlamydomonas sp. (W22). All of these forms were grown upon the medium used by Wann:

*Obtained through the courtesy of Dr. Frank B. Wann of the Utah Agricultural College

NH_4NO_3	0.05 %
K_2HPO_4	0.02 %
$MgSO_4$	0.01 %
$CaCl_2$	0.01 %
$FeSO_4$	trace
Glucose	1.00 %
Agar	1.50 %
Distilled water	

All cell suspensions and serum dilutions were made with 0.9 per cent sodium chloride solution, in which the euglenas will remain alive in an active condition for several days. The euglenas, moreover, seem unaffected by an exposure of several hours to a temperature of 37°C. , though a temperature only a few degrees above this is fatal to them.

For the production of an immune serum five rabbits were given intraperitoneal injections of the living cells of Euglena gracilis (Noland strain,light, except in the case of one rabbit in which immunization had been begun with a culture grown in the dark). The rabbit whose serum showed the greatest immunity had received injections of 0.5 cc. ,1.0 cc. , 1.5 cc. , and 1.5 cc. (volumes are those of packed cells) at intervals of five, seven, and seven days respectively. The large size of the cells did not permit intravenous injections. Treatment of a rooster was discontinued after the third injection, for no response could be detected. The injected material was not toxic.

Two rabbits were given repeated intraperitoneal and intravenous injections of Stichococcus sp. , three of Chlorella sp. (W5), and one of Chlamydomonas sp.

Antigen-antibody Reactions Studied

Chlorolysis. It had occasionally been noted with euglenas that had stood for some time in low serum dilution that the supernatant fluid was tinged with green. This was not obtained consistently, and was finally correlated with a serum concentration sufficiently high to kill the cells. Upon substituting euglenas that had been killed by exposure to a temperature of 45° C. for ten minutes, this result was regularly obtained with either fresh or inactivated rabbit serum. It was not increased by immunization. The effect was noticeable within an hour at 37° C. in a 1:10 serum dilution, but did not extend much further than this. It was never complete enough so that microscopic examination of the cells could have detected it. The only other serum tested was that of guinea pig, which acted in the same way. These facts are summarized in table 1. Results were negative with normal rabbit serum and either Stichococcus or Chlorella heated to the same extent as the euglenas, though it was not determined whether these cells were killed at this temperature. Sherwood [25] has described a similar

Table 1

Chlorolysis resulting from the action of serum upon a heat-killed suspension of *Euglena gracilis*

0.5 cc. dilution of serum		0.5 cc. of 1:5 cell suspension (heated 10 minutes at 45° C.)	Result*
Rabbit, normal, fresh	1:5	"	###
" " "	1:10	"	##
" " "	1:25	"	#
" " "	1:50	"	-
Rabbit, normal, heated	1:5	"	###
" " "	1:10	"	##
" " "	1:25	"	#
" " "	1:50	"	-
Rabbit, immune, fresh	1:5	"	###
" " "	1:10	"	##
" " "	1:25	"	#
" " "	1:50	"	-
Guinea pig, fresh	1:5	"	###
" "	1:10	"	##
" "	1:25	"	#
" "	1:50	"	-
Guinea pig, heated	1:5	"	###
" "	1:10	"	##
" "	1:25	"	#
" "	1:50	"	-

* ###, chlorolysis marked
##, chlorolysis less marked
#, chlorolysis hardly detectable
-, supernatant liquid clear

chlorolysis of the chloroplasts of certain higher plants, which was brought about by the normal serum of various animals together with plant extracts.

Agglutination. With the serum of eleven normal rabbits studied, slight agglutination of Euglena gracilis was only occasionally, and then not consistently, obtained in low dilution. The occurrence of normal agglutinins for this species was also studied in the sera of the following: human (2), guinea pig (2), beef (3), chicken (2), dog (1), frog (2), and salamander (1). The results were not greatly different from those with rabbit serum, though in some, particularly beef, distinct agglutination occurred in low dilution.

Agglutination tests with immune serum were never satisfactory. With anti-euglena serum, complete agglutination, consisting in the formation of soft clumps, was often observed in the 1:10* dilution. Above this dilution its occurrence was doubtful.

The serum of rabbits injected with Stichococcus sp. caused complete agglutination of Stichococcus in a 1:10 dilution with partial agglutination extending to a 1:100 dilution. The serum of a rabbit given thirteen daily intravenous injections of 0.1 cc.

*Serum dilutions refer to original dilutions.

(packed cells) of Chlorella sp. (W5) completely agglutinated this form in a 1:50 dilution with partial agglutination extending to a 1:250 dilution. The Chlamydomonas-immune rabbit serum had a titer of 1:10. No group reactions were detected with these low-titered sera.

Cytotoxin Reaction. An attempt was made to determine the occurrence of natural cytotoxins for Euglena gracilis in the serum of the series of normal animals mentioned above. Fresh serum of normal rabbits up to a 1:10 dilution when mixed with an equal volume of a suspension of the euglenas caused their complete sedimentation, from which they did not later recover, during an incubation period of one hour at 37°C. In a 1:10 dilution the flagellum was discarded within a few minutes after contact with the serum. The euglenas were not killed in normal rabbit serum (except perhaps in undiluted serum), but retained their characteristic euglenoid movement. Partial sedimentation occasionally extended to a 1:50 serum dilution, but was followed by complete recovery within twenty-four hours (often much less) at room temperature. The toxic effect of fresh normal serum was nearly destroyed by heating one-half hour at 56°C.

The toxicity of the fresh serum of the other

animals studied seemed in all cases to extend to a slightly higher serum dilution than in the case of rabbit serum, but the series run was not extensive enough to warrant drawing conclusions as to species differences in this regard.

This property of serum to cause complete sedimentation of the euglenas was increased upon immunization. It extended quite uniformly in the rabbits tested to a 1:1000 serum dilution, and further immunization did not increase it. The effect could be detected macroscopically in the lower dilutions within fifteen minutes at 37° C. , and all of the tubes could be "read" at the end of an hour's incubation. In the 1:100 and higher serum dilutions complete recovery frequently took place. The sedimentation titer of the serum was not altered by heating the serum one-half hour at 56° C. but gradually dropped in preserved or frozen serum. A typical titration is shown in table 2.

The process of sedimentation and recovery was watched in hanging drop preparations with various serum dilutions. The flagellum was discarded, as with fresh normal serum, up to the limiting dilution of sedimentation. This took place almost immediately in the lower dilutions, after a longer time in higher dilutions (in about three minutes in a 1:100 dilution). The

Table 2

Sedimentation of *Euglena gracilis* by normal and by homologous immune rabbit serum

0.5 cc. dilution of serum	0.5 cc. culture of *Euglena gracilis*	Degree* sedimentation	
		1 hour 37° C.	24 hours
0.9 % NaCl	Noland, light	-	-
Normal			
fresh 1:10	" "	####	####
1:20	" "	##	-
1:50	" "	-	-
heated 1:10	" "	-	-
Immune, 1:50	" "	####	### (often)
fresh 1:100	" "	####	-
or 1:250	" "	####	-
heated 1:500	" "	####	-
1:1,000	" "	####	-
1:2,000	" "	#	-
1:5,000	" "	-	-

* ####, complete sedimentation
###, nearly complete sedimentation
##, partial sedimentation
#, slight sedimentation
-, uniform distribution

cell body usually became immobile, while the flagellum continued in vigorous motion for some time, until it suddenly separated from the cell. A knob-shaped enlargement (the thickening at the opening of the reservoir ?) was seen at one end of the free flagellum. Recovery was accompanied by the formation of a new flagellum. This took place comparatively quickly, being well under way within two hours in a 1:100 serum dilution, as shown by the following protocol:

1:50 inactivated immune rabbit serum

Flagella thrown off immediately; non-motile contracted forms. Little change after two hours; some show euglenoid movement.

1:100 inactivated immune rabbit serum

5:12 P.M. - Culture of Euglena added to equal amount of serum dilution.
5:15 P.M. - Flagellum of organism being watched under microscope discarded.
6:50 P.M. - Vibratory motion visible at anterior end.
7:00 P.M. - Metaboly resumed.
7:20 P.M. - New flagellum well started.

1:50 inactivated normal rabbit serum

No change. Swimming actively.

0.9 % NaCl

No change. Swimming actively.

As cessation of motility preceded the flagellum expulsion, it is probable that in the higher dilutions

many may drop to the bottom and recover without throwing off the flagellum.

All attempts to determine whether the recovered organisms were more resistant to a second exposure to the same serum dilution were unsatisfactory. Sedimentation occurred in the control tubes from the repeated handling and centrifuging, and bacterial contamination.

Reference to table 3 shows that some adsorption of the sedimentation factor of immune serum was accomplished.

No difference in the sedimentation titer could be detected when the Turtox strain of Euglena gracilis was used with the Noland strain immune serum. The titers also were the same when the green and colorless forms of the Noland strain were used with the Noland strain green immune serum; results with a low titered serum to the dark form were not so distinct.

Complement Fixation. Complement fixation tests with Euglena-immune serum were performed in only one instance. The serum, which was that of a rabbit given four injections, caused complete sedimentation as described above in 1:2000 dilution. The anticomplementary unit of the serum was 0.3 cc. of a 1:10 dilution. The test was negative.

The serum of a rabbit given many injections of

Table 3

Sedimentation of Euglena gracilis by the homologous immune rabbit serum after adsorption with Euglena gracilis, and after adsorption with Chlorella sp. (W11)

0.5 cc. dilution of serum adsorbed with		0.5 cc. culture of Euglena gracilis	Degree* sedimentation 1 hour, 37° C.
Euglena	1:50	"	###
"	1:100	"	###
"	1:250	"	###
"	1:500	"	-#
"	1:1000	"	-#
"	1:5000	"	-
0.9 % NaCl		"	-
Chlorella	1:50	"	####
"	1:100	"	####
"	1:250	"	####
"	1:500	"	###
"	1:1,000	"	##
"	1:5,000	"	##

* #### complete sedimentation
nearly complete sedimentation
partial sedimentation
slight sedimentation
-# any sedimentation doubtful
- uniform distribution

Stichococcus, and that of a rabbit treated similarly with Chlorella were found to be anticomplementary, when 0.1 cc. of a 1:10 dilution was used.

Precipitation. The serum of three of the rabbits injected with Euglena was tested for the presence of precipitins, using as antigen a protein extract prepared according to the method of Besredka [2]. One was clearly negative, one positive with the undiluted extract, and one positive in 1:10 dilution of the extract. The protein concentration of the extract is unknown.

The serum of a rabbit immunized to Stichococcus showed a precipitate with 1:50 dilution of Besredka extract. A precipitate with undiluted Besredka extract was produced by the serum of a rabbit immunized to Chlorella (W5).

Anaphylaxis. Work has been begun upon anaphylaxis with these organisms. It is as yet incomplete, and is not reported upon here. The method of Dale [6] in particular seems to offer a promising field for further work, which will be continued in this direction.

DISCUSSION

In explanation of the low degree of immunity obtained, several possibilities suggest themselves.

Any antigenic properties the cells possess must be due to the proteins they contain, and it may be that these, even were they all antigenic, are present in too small amount for the quantities injected to produce much effect. Complete chemical analyses of the forms studied are not available. Determinations by various workers differ widely and are doubtless influenced by the age and substrate of the cultures used. The following data concerning Chlorella vulgaris are supplied by Fred and Peterson [9]. The ash constituted 17.6 per cent of the dry weight (this high value explained by retention of salts of medium). The water-soluble nitrogen precipitated by Folin-Wu's tungstic acid reagent (a complete deproteinizer) represented 0.531 per cent of the dry ash-free material. If all of this is protein nitrogen, and therefore approximately 16 per cent of the total protein, the dry ash-free cells contained 3.3 per cent protein. Nicolle and Alilaire [15] give the moisture determination for Chlorella vulgaris as 63.6 per cent.

That physical structure as well as chemical constitution may have much to do with the poor antigenic capacity of the whole cells of the organisms used was suggested by a recent article by Zirkle [30] on the structure of the chloroplast. He found that

chloroplasts of higher plants from which the chlorophyll had been removed by solvents were digested by proteolytic enzymes, while those containing even a trace of chlorophyll were unaffected. He suggests that this may be due to the distribution of the chlorophyll on the surface of the colloidal particles of the stroma in such a way as to prevent the enzymes from reaching the ground substance. If this physical structure holds true in the case of the organisms used, in which the chloroplasts constitute no inconsiderable part of the cell, the chlorophyll may protect the protein constituents of the chloroplasts from contact with the body cells, and thereby prevent antibody formation, even though the isolated proteins might be highly antigenic. Wells [29] notes that proteins which are indigestible by enzymes are seldom antigenic.

If it were true that only the cytoplasm without the chloroplasts is the antigenic portion of the cell, the greater immunity produced to the euglenas than to the other forms used could readily be accounted for by the greater amount of cytoplasm outside of the chloroplasts. On the other hand, it may be that the greater immunity apparently produced with the euglenas is merely due to a more delicate means of its detection. The amount of antibody substance in the circulation might

be negligible, and this small amount identical with the known antibodies, the reason for sedimentation being the great sensitiveness of the cell to these substances. The results of quantitative complement fixation tests would be of interest in this connection.

That solubility in the body fluids, a necessary condition for antibody formation, is not possessed to a high degree by the whole cells used is shown by the following observation. At autopsy of two rabbits that had received the last intraperitoneal injection of euglenas more than a week before, there was found in the body cavity considerable unabsorbed material in which the form of the cells could still be distinguished upon microscopic examination. The presence of a resistant cell wall may be the determining factor in this. However, in the case of euglenas at least, the cell membrane is sufficiently permeable for slow chlorolysis to occur from dead organisms in contact with serum. It is a well-known fact that many ciliates and amebas undergo an explosive disintegration upon disturbance of their normal osmotic relationships. In these cases, even though the formed cells were injected they would burst upon contact with the body fluids, at once exposing an immensely greater surface to the tissues. It is only to be expected that greater

antibody formation would ensue here than in the case of the more slowly disintegrated euglenas.

Little has been found in the literature concerning the significance of the throwing off of the flagellum by euglenas. It is probable that it is merely a general injury reaction which can be produced by many substances. Fischer [8] held that the flagella of all flagellates are invariably discarded upon irritation. With Euglena gracilis it is regularly thrown off (or absorbed ?) preceding encystment and cell division. In the case of the serum there is no possibility of absorption. Zumstein [31] describes the phenomenon in his discussion of the effect of various organic acids upon Euglena gracilis:

"Kurz nach dem übertragen in die Säure sinken die Euglenen langsam auf dem Boden des Versuchsgläschems, denselben mit einer Kruste überziehend. Ihre Schwimmbewegung wird sistirt, die Geissel abgeworfen und viele Individuen sterben schneller oder langsamer ab. War aber Säure nicht zu stark, so erholen sich manche der scheintodten Euglenen wieder, bilden ihre Cilien von von neuem aus und vertheilen sich gleichmässig in der ganzen ihnen zu Gebote stehenden Flüssigkeit. "

A comparison of this description with that given above of the effect of immune serum shows that no effects can be attributed to the latter that could

not be attributed to other substances of equal toxicity.

It is possible that this immunity manifested by sedimentation is merely an intensification of the normal serum property described above, but the heat-sensitiveness of the latter would suggest that the toxicity of normal serum is due largely to its complement content, while the thermostability of the former would connect it rather with the known thermostable antibodies.

The nature of the immune body which produces sedimentation of the euglenas needs further study. Rous' [23] work would tend to show that there may be a specific toxic substance in immune serum entirely apart from the other antibodies. He found that a high-titered anti-guinea pig rabbit serum was highly toxic to guinea pigs, and that the toxicity was not decreased by the removal of all hemolysins, hemagglutinins, and precipitins. It might be that such an antibody is commonly produced, but that the conditions of testing for immunity preclude its detection.

It is true of the resistance of all organisms to toxic substances that great individual variations occur. This has been well discussed by Peters [17] and others. With unicellular forms those individuals of smaller size or with more delicate cell membrane might

be the first ones affected, due to the more rapid penetration of the injurious material. The degrees of sedimentation of the euglenas noted are readily explained by individual variation.

Some inconsistencies in the results with agglutination tests with Stichococcus and Chlorella suggested that the age of the culture might have considerable influence upon the inagglutinability of these forms. It is possible that the method of Mudd [14], in which the binding of agglutinins is detected by the difficulty with which the cells are resuspended after sedimentation, might find application here.

It is possible that the production of sedimentation, which was the most apparent property of euglena-immune rabbit serum, might be of value in a comparison of the antigenic relationships of the forms included in the order Euglenida. However, it is obvious that it could not find application with those euglenas which do not swim freely, and it might be that differences in the sensitiveness of closely related organisms to this property would not be correlated with antigenic differences. It may be anticipated here, from a comparison of the work reported upon above with green and colorless forms of the same organism with that in progress, that the smooth muscle anaphylactic reaction will be found

to be of greater delicacy and wider applicability than the sedimentation phenomenon.

SUMMARY

1. A slight chlorolysis occurred with a dead suspension of Euglena gracilis in contact with a 1:10 dilution of fresh or inactivated normal rabbit or guinea pig serum.

2. This effect was not increased upon immunization.

3. Beef serum seemed to have a greater agglutinating effect for Euglena gracilis than other normal sera.

4. Immunization with Euglena gracilis does not readily lead to the production of agglutinins for this form.

5. Fresh normal serum of rabbit, and other species studied, caused complete sedimentation without later recovery of Euglena gracilis. Partial sedimentation with complete recovery occasionally extended to a 1:50 serum dilution.

6. The toxic effect of fresh normal sera for Euglena gracilis was nearly destroyed by heating one-half hour at 56°C.

7. The serum of rabbits given intraperitoneal injections of the whole cells of Euglena gracilis caused complete sedimentation of the euglenas without recovery (usually) in a 1:50 dilution, and complete sedimentation with complete (often) recovery in a 1:1000 dilution.

8. The sedimentation of Euglena gracilis by high concentration of normal and by high or low concentration of immune serum was accompanied by the discarding of the flagellum, the recovery by its regeneration.

9. The effect of the immune serum was not altered by heating one-half hour at 56°C.

10. There was no difference in the sedimentation titer of the immune serum when tested with another strain of Euglena gracilis, or with a colorless form, produced by growth in the dark, of the same strain.

REFERENCES

1. Barber, M. A. - The pipette method in the isolation of single microorganisms and in the inoculation of substances into living cells. Philippine Journal of Science B 9 (4): 307-360. 1914.

2. Besredka - Des endotoxines solubles typhique, pesteuse et dysenterique. Annales de l'Institut Pasteur 20 (4): 304-310. 1906.

3. Bills, C. E. - The preparation of five kilograms of desiccated Euglena. Science n.s. 57 (1487): 740-741. 1923.

4. Calkins, G. N. - The biology of the Protozoa. Philadelphia: Lea and Febiger. p. 192. 1926.

5. Coca, A. F. - The separation of protozoan species by means of immunity reactions. Zeitschrift für Immunitätsforschung und experimentelle Therapie 12 (2): 127-133. 1912.

6. Dale, H. H. - The anaphylactic reaction of plain muscle in the guinea pig. Journal of Pharmacology and Experimental Therapeutics 4 (3): 167-224. 1913.

7. Ehrlich, P. - Über Partialfunktionen der Zelle. Münchener medizinische Wochenschrift 56 (5): 217-222. 1909. Cited by Taliaferro [27].

8. Fischer, A. - Über die Geisseln einiger Flagellaten. Jahrbücher wissenschaftliche Botanik 27 : 187-235. 1894. Cited by Calkins, G. N. - The Protozoa. New York: Macmillan. p. 120. 1910.

9. Fred, E. B. , and Peterson, W. H. Forms of nitrogen in pure cultures of algae. Botanical Gazette 79 (3): 324-328. 1925.

10. Heathman, L. - Local citation.

11. Kowarski - Über den Nachweiss von pflanzlichen Eiweiss auf biologischen Wege. Deutsche medizinische Wochenschrift 27. 1901. Cited by Steinecke, F. - Die Sero-Diagnostik als Hilfsmittel botanischer Verwandtschaftsforschung. Der Naturforscher (Schoenichen) 2 (1): 1-5. 1925.

12. Levaditi, C. , and McIntosh - Méchanisme de la création de races de trypanosomes résistantes aux anticorps. Bull. Soc. Path. Exot. 3: 368-376. 1910. Cited by Taliaferro 27.

13. Lieske, R. - Serologische Studien mit einzelligen Grünalgen. Sitzungsberichte der Heidelberger Akademie der Wissenschaften B, Abhandlung 3, 1-47. 1916.

14. Mudd, S. - A simple reaction for detecting the binding of agglutinins by difficultly agglutinable suspensions. Journal of Immunology 13 (2): 113-121. 1927.

15. Nicolle, M. , and Alilaire, E. - Note sur la production en grand des corps bactériens et sur leur composition chimique. Annales de l'Institut Pasteur 23 (7): 547-557. 1909.

16. Noguchi, H. - Comparative studies of herpetomonads and leishmanias. II. Differentiation of the organisms by serological reactions and fermentation tests. Journal of Experimental Medicine 44 (3): 327-357. 1926.

17. Peters, R. A. - Variations in the resistance of protozoan organisms to toxic agents. Journal of Physiology 54 (4): 260-266. 1920.

18. Pringsheim, E. G. - Kulturversuche mit chlorophyllführenden Mikroorganismen. II. Zur Physiologie der Euglena gracilis. Beitrage zur Biologie der Pflanzen 12 (1): 1-48. 1913.

19. Rössle, R. - Spezifische Sera gegen Infusorien. Archiv für Hygiene 54: 1-31. 1905.

20. Rosenblat-Lichtenstein, S. - Über die Differenzierung von Algen mit Hilfe spezifischer Agglutinine. I. Archiv für Physiologie (Physiologische Abteilung des Archives für Anatomie und Physiologie) 5-6: 415-420. 1912

21. Rosenblat-Lichtenstein, S. - Agglutination bei Algen. II. Beziehungen des Stoffwechsels der Zelle zu ihrem agglutinatorischen Verhalten. Archiv für Physiologie (Physiologische Abteilung des Archives für Anatomie und Physiologie) 1-2: 95-99. 1913.

22. Rosenthal, F. - Untersuchungen über die Genese des Rezidivs bei der experimentellen Trypanosomeninfektion. Zeitschrift für Hygiene und Infektionskrankheiten 74: 489-538. 1913. Cited by Taliaferro [27].

23. Rous, P. , Wilson, G. W. , and Oliver, J. - Experiments on the production of specific antisera for infections of unknown cause. III. The effects of a serum precipitin on animals of the species furnishing the precipitinogen. Journal of Experimental Medicine 31 (3): 253-265. 1920.

24. Sellards, A. W. - Immunity reactions with amebas. Philippine Journal of Science B 6: 281-298. 1911.

25. Sherwood, M. P. - A study of the antigenic properties of chlorophyll. Abstracts of Bacteriology 9 (1): 22. 1925.

26. Steinecke, F. - Der Stammbaum der Algen nach serodiagnostischen Untersuchungen dargestellt. Botanisches Archiv 10 (1-2): 82-205. 1925.

27. Taliaferro, W. H. - Host resistance and types of infections in trypanosomiasis and malaria. Quarterly Review of Biology 1 (2): 246-269. 1926.

28. Ternetz, C. - Beiträge zur Morphologie und Physiologie der Euglena gracilis Klebs. Jahrbücher für wissenschaftliche Botanik 51 (4): 435-514. 1912.

29. Wells, H. G. - Chemical Aspects of Immunity. New York: Chemical Catalog Company. pp. 40-41. 1925.

30. Zirkle, C. - The structure of the chloroplast in certain higher plants. American Journal of Botany 13 (5,6): 301-341. 1926.

31. Zumstein, H. - Zur Morphologie und Physiologie der Euglena gracilis Klebs. Jahrbücher für wissenschaftliche Botanik 34: 149-196. 1900.